

エナメルマトリックス誘導体はリポ多糖で刺激された
ヒト顎堤粘膜由来血管内皮細胞において免疫応答を誘導する

八板 直道

Enamel matrix derivative induces an immune response in human alveolar ridge
mucosa-derived vascular endothelial cells stimulated with lipopolysaccharide

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ことを承諾します。

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Enamel matrix derivative induces an immune response in human alveolar ridge mucosa-derived vascular endothelial cells stimulated with lipopolysaccharide

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【Abstract】

Background

Early detection of peri-implant disease is difficult. Enamel matrix derivative (EMD), which is used for periodontal tissue regeneration, reportedly promotes the gene expression of leukocyte chemotactic factors and adhesion molecules in vascular endothelial cells. Therefore, we hypothesized that stimulating vascular endothelial cells with EMD would induce an inflammatory response in the peri-implant mucosa, allowing for the early detection of peri-implant infection. To verify this hypothesis, we evaluated the gene expression levels of leukocyte chemotactic factor and cell adhesion molecule, in addition to assessing intercellular adhesion in human alveolar ridge mucosa-derived vascular endothelial cells (ARMEC) stimulated with lipopolysaccharide (LPS) and EMD.

Methods

We established an experimental model of peri-implant disease by stimulating ARMEC, representing the peri-implant mucosa, with *Porphyromonas gingivalis*-derived LPS. ARMEC were obtained from patients (n = 6) who visited The Nippon Dental University Niigata Hospital, Japan. The cells were stimulated with LPS (1 µg/mL) and/or EMD (100 µg/mL), and cell viability, gene expression levels of leukocyte chemotactic factor (interleukin-8 : *IL-8*), adhesion molecule (intercellular adhesion molecule-1 : *ICAM-1*) and tight junction proteins gene expression (zonula occludens-1 : *ZO-1* and *Occludin*), and trans-endothelial electrical resistance (TEER) were measured.

Results

LPS reduced ARMEC viability, whereas simultaneous stimulation with EMD improved cell viability. Stimulation with LPS and EMD enhanced *IL-8* and *ICAM-1* gene expression levels, suppressed TEER, and decreased *ZO-1* and *Occludin* gene expression levels compared to stimulation with LPS alone.

Conclusion

EMD may stimulate leukocyte migration, increase vascular permeability, and trigger an immune response in the peri-implant mucosa, thus facilitating the early detection of peri-implant disease and allowing for early treatment intervention.

【Background】

Peri-implant diseases are inflammatory conditions that affect the tissues around dental implants.¹ They are broadly categorized into peri-implant mucositis, characterized by localized inflammation in the peri-implant mucositis, and peri-implantitis, which involves bone destruction.² In peri-implant tissues, blood is supplied via vessels originating in the peri-implant mucosa and alveolar bone periosteum, unlike in periodontal tissues, which have periodontal ligament arteries.³ Peri-implantitis progresses more rapidly than periodontitis, and the inflammation is more extensive.^{4,5} Therefore, detecting early signs of peri-implant diseases is crucial for preventing aggravation. However, identifying initial peri-implantitis lesions can be challenging, as the inflammatory signs in the peri-implant mucosa may not be as clear as those in periodontitis, making early detection difficult.⁶

Endothelial cells lining the vessel lumen play a role in regulating leukocyte migration and endothelial permeability through the gene expressions of interleukin-8 (*IL-8*) and intercellular adhesion molecule-1 (*ICAM-1*), as well as through cell-cell adhesion at tight junctions.⁷⁻¹⁰ However, the characteristics of endothelial cells in the blood vessels of the peri-implant mucosa are unknown.

Enamel matrix derivative (EMD) is a material primarily composed of enamel matrix proteins extracted from the tooth germ of young pigs. EMD is used for regenerating periodontal tissues in periodontal surgical treatments. It induces the differentiation of undifferentiated mesenchymal cells into cementoblasts, thereby regenerating the acellular cementum.¹¹ Furthermore, the EMD affects cells other than cementoblasts. For example, Villa et al. reported that EMD influences IL-8 gene expression in human periodontal ligament fibroblasts.¹² In addition, Bertl et al. found that EMD affects *ICAM-1* gene expression and angiogenesis in human umbilical vein endothelial cells (HUVEC).¹³ However, the effects of EMD on the vascular endothelial cells of the peri-implant mucosa have not been reported to date.

With the goal of early detection and prevention of peri-implant mucositis, in this study, we here explored the potential of inducing an immune response by stimulating human alveolar ridge mucosa-derived vascular endothelial cells (ARMEC) with EMD. To this end, we analyzed gene expression levels of leukocyte chemotactic factor, adhesion molecule and cell-cell adhesion in ARMEC, which assumed in the peri-implant mucositis, that had been stimulated with lipopolysaccharide (LPS) to induce inflammation.

【Methods】

Cell isolation and culture

Alveolar ridge mucosal samples were obtained from patients who visited The Nippon Dental University Niigata Hospital and were scheduled for implant placement ($n = 6$, three males, three females, mean age, 44.2 ± 9.2 years). Sites where implant treatment would be conducted were targeted, and the samples were collected from tissues excised during the implant placement. The above-mentioned method was adopted because it has previously been reported that removing healthy peri-implant mucosa after implant placement influences the prognosis of implant treatment, making it difficult to collect healthy human peri-implant mucosa.¹⁴ The patients included in the study had no systemic diseases and were non-smokers. The harvested tissues were cut into 1-mm^3 sections, placed into 35-mm culture dishes (BD BioCoat[®]; Becton Dickinson Biosciences, Franklin Lakes, NJ, USA), and allowed to settle. Then, Dulbecco's modified eagle's medium/Nutrient Mixture F-12 (Invitrogen, Waltham, MA, USA) supplemented with 15% fetal bovine serum (FBS; JR Scientific, Woodland, CA, USA), 100 U/mL penicillin (Invitrogen), 100 $\mu\text{g/mL}$ streptomycin (Invitrogen), and 250 $\mu\text{g/mL}$ amphotericin B (Invitrogen) were added. The primary cultures were incubated at 37°C in the presence of 5% CO_2 95% air. After cell outgrowth from the tissue sections was confirmed, the culture medium was changed every other day, and the cells were cultured for approximately 2 weeks until they reached confluence. Vascular endothelial cells were isolated from the cultured alveolar ridge mucosa and periodontal ligament cells using the method described by Tsubokawa et al.¹⁵ The cultured cells were collected, and anti-CD31 antibody-coated magnetic beads (Dynabeads[®] CD31 Endothelial Cells; Invitrogen) were added. Subsequently, ARMEC were isolated using a magnet (DynamagTM-15; Invitrogen). Periodontal ligament-derived vascular endothelial cells (PDLEC) and HUVEC (Lonza, Walkersville, MD, USA) were used as controls. Periodontal ligament samples were harvested from extracted teeth of patients ($n = 6$, including three males and three females; mean age, 42.8 ± 13.6 years) who visited The Nippon Dental University Niigata Hospital for treatment. PDLEC were isolated using the same method as that used for ARMEC. ARMEC, PDLEC and HUVEC were cultured in Endothelial Cell Growth Medium-2 BulletKit[™] (EGM-2; Lonza) containing 5% FBS. ARMEC, PDLEC and HUVEC had a passage number of 3.

This study was approved by the Ethics Review Committee of the School of Life Dentistry at Niigata, The Nippon Dental University (approval number: ECNG-R-390). This study was conducted in accordance with the guidelines of the Declaration of Helsinki, with written informed consent obtained from each participant.

Cell culture conditions

To examine vascular endothelial cells during inflammatory reactions, cells were stimulated with LPS derived from *Porphyromonas gingivalis* (InvivoGen, San Diego, CA, USA). LPS was added to EGM-2 at a concentration of 1 µg/mL. To investigate the effects of EMD on ARMEC and PDLEC during inflammation, we used Emdogain® Gel (Straumann, Basel, Switzerland). Emdogain® Gel was 30 mg/ml, and it was diluted with EGM-2 to a concentration of 100 µg/ml and used as an EMD medium. Separate cultures were tested using a medium containing only LPS, a medium containing only EMD, and another medium containing both LPS and EMD. The experimental groups included an LPS-stimulated group (LPS) cultured in LPS-supplemented medium, an EMD-stimulated group (EMD) cultured in EMD-supplemented medium, and an LPS + EMD-stimulated group (LPS + EMD) cultured in medium supplemented with both LPS and EMD. A non-stimulated control group (control) was cultured in non-supplemented medium. For ARMEC, the LPS, EMD, and LPS + EMD groups were designated as ARMEC + LPS, ARMEC + EMD, and ARMEC + LPS + EMD, respectively. Similarly, for PDLEC, the corresponding groups were labeled PDLEC + LPS, PDLEC + EMD, PDLEC + LPS + EMD, respectively.

Cell viability assay

ARMEC and PDLEC were seeded in a 96-well plate (Becton Dickinson Biosciences) at 1.5×10^3 cells/100 µL and were cultured in EGM-2 for 24 h. Then, the medium was exchanged for treatment or control medium and the cells cultured for 10 days. After 1, 2, 4, 6, 8, and 10 days of culture, cell viability was assessed by mitochondrial reduction staining by using alamarBlue™ Cell Viability Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and measuring the fluorescence intensity using a microplate reader (POWERSCAN MX; DS Pharma Biomedical, Osaka, Japan).

Gene expression analysis of leukocyte migration factors and tight junction proteins

ARMEC, PDLEC and HUVEC were seeded in 60-mm dishes (BD BioCoat®; Becton Dickinson Biosciences) at 1.0×10^5 cells/mL and cultured in EGM-2. The gene expression levels of *IL-8* and *ICAM-1* were analyzed after 6 h of culture and those of tight junction proteins (zonula occludens-1 : *ZO-1* and *Occludin*) were analyzed after 6 days of culture, using reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cells using NucleoSpin® RNA (Takara Bio, Shiga, Japan). cDNA was synthesized from 1 µg of RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Fifty nanograms of cDNA was mixed with 5 µM forward and reverse primers (Fasmac, Kanagawa, Japan) and 25 µL Power SYBR® Green PCR Master Mix (Applied Biosystems) in a 96-well plate (FrameSter® FastPlate 96; 4titude, Surrey, UK). RT-qPCR was carried out using a StepOnePlus Real-time PCR System

(Applied Biosystems). The PCR conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 15 s of denaturation at 95°C, 1 min of annealing at 60°C and 15 s of extension at 95°C. Relative gene expression levels were determined using the comparative cycle threshold ($\Delta\Delta C_t$) method in StepOne™ software (version 2.2; Applied Biosystems). The glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) was used as an internal control. The primer sequences for each gene are listed in Table 1.

Trans-endothelial electrical resistance measurement

ARMEC and PDLEC were seeded in a 24-well plate (Millipore, Billerica, MA, USA) at 1.0×10^4 cells/100 μ L and cultured in EGM-2 for 24 h. Then, the medium was exchanged for treatment or control medium. The cells were cultured for 6 days, and the resistance of the vascular endothelial cell monolayers was measured every 48 h using a Millicell® ERS-2 electrical resistance system (Millipore). TEER ($\Omega \text{ cm}^2$) was calculated by subtracting the resistance value of the blank sample from the measured resistance value of each vascular endothelial cell monolayer and multiplying this value by the membrane surface area (0.6 cm^2).

Statistical analysis

If the data showed a normal distribution according to the Shapiro-Wilk test, one-way analysis of variance was used, and post hoc multiple comparisons were conducted using the Tukey-Kramer test. Indications of non-normal distribution were performed using the Kruskal-Wallis test, and post hoc multiple comparisons were conducted using the Steel-Dwass test. Statistics between two groups in Fig. 4B, D was performed using a t-test. Statistical significance was set at $p < 0.05$. Mac Statistical Analysis Version 3.0 (Ezumi, Tokyo, Japan) was used for statistical analysis.

【Results】

Effects of LPS and EMD stimulation on cell proliferation

Cells in all stimulated groups showed an increase in cell proliferation over time (Fig. 1A). On day 10, cell proliferation was significantly lower in ARMEC + LPS than in ARMEC ($p < 0.05$), whereas that of ARMEC + EMD was significantly higher than that of ARMEC ($p < 0.05$). The cell proliferation in the ARMEC + LPS + EMD group was not significantly different from that of ARMEC, whereas that of ARMEC + EMD was significantly higher than that of ARMEC + LPS ($p < 0.01$). The cell proliferation of ARMEC + EMD was not significantly different from that of ARMEC + LPS + EMD (Fig. 1B).

Effects of LPS and EMD stimulation on gene expression levels for leukocyte migration factors after 6 h of treatment

Gene expression levels were presented relative to that in non-stimulated HUVEC at 6 h of culture in Fig 2, 3 A-D and relative to that in non-stimulated ARMEC at 6 h of culture in Fig 2, 3 E and F (* $p < 0.05$, ** $p < 0.01$). *IL-8* and *ICAM-1* gene expression levels were significantly increased in both ARMEC + LPS and ARMEC + EMD ($p < 0.05$). ARMEC + LPS exhibited significant decreases in *IL-8* and *ICAM-1* gene expression levels as compared to PDLEC + LPS ($p < 0.01$), whereas those of ARMEC + EMD did not differ significantly as compared to PDLEC + EMD. ARMEC + LPS exhibited significantly lower *IL-8* and *ICAM-1* gene expression levels than ARMEC + LPS + EMD ($p < 0.01$, $p < 0.05$, respectively). ARMEC + LPS + EMD showed no significant differences in *IL-8* or *ICAM-1* gene expression levels as compared to PDLEC + LPS (Fig. 2A-F).

Effects of LPS and EMD stimulation on gene expression levels for tight junction proteins on day 6

ZO-1 and *Occludin* gene expression levels were significantly higher in ARMEC than in PDLEC ($p < 0.05$). These levels were decreased in ARMEC + LPS and ARMEC + EMD compared to ARMEC. *ZO-1* and *Occludin* gene expression levels in ARMEC + LPS + EMD was decreased significantly as compared to ARMEC + LPS ($p < 0.05$). *ZO-1* and *Occludin* gene expression levels did not differ significantly between ARMEC + LPS + EMD and PDLEC + LPS (Fig. 3A-F).

Effects of LPS and EMD stimulation on TEER

Cells in all treatment groups showed a gradual increase in TEER over time (Fig. 4A). On day 6, TEER was significantly higher in ARMEC than in PDLEC ($p < 0.01$) (Fig. 4B). TEER of ARMEC + LPS, ARMEC + EMD and ARMEC + LPS + EMD were significantly lower than that of ARMEC ($p < 0.01$, $p < 0.05$), whereas that of ARMEC + LPS was not significantly different from that of ARMEC + EMD. ARMEC + LPS had significantly higher TEER than did ARMEC + LPS + EMD ($p < 0.05$), whereas that of ARMEC + EMD did not differ significantly from that of ARMEC + LPS + EMD (Fig. 4C). TEER of ARMEC+LPS+EMD was not significantly different from that of PDLEC+LPS (Fig. 4D).

【Discussion】

This study aimed to assess the expression of leukocyte chemotactic factors and cell adhesion molecules, along with examining intercellular adhesion in ARMECs stimulated with LPS and EMD. The investigation aimed to explore the potential for early detection and prevention of peri-implant mucositis. LPS reduced ARMEC viability, but simultaneous stimulation with EMD improved cell viability, enhanced *IL-8* and *ICAM-1* gene expression levels, suppressed TEER, and decreased *ZO-1*

and *Occludin* gene expression levels. EMD may stimulate leukocyte migration and adhesion molecules, increase vascular permeability and trigger an immune response in the peri-implant mucosa, thus facilitating the early treatment detection of peri-implant disease and allowing for early intervention. The ARMEC used in this study were isolated from tissues excised during implant placement. This approach was chosen to prevent potential influences on the morphology of the peri-implant mucosa and to ensure samples with sufficient thickness are obtained. This is crucial as obtaining samples from the healthy peri-implant mucosa of already placed implants could be challenging.¹⁴ Peri-implant and periodontal diseases are associated with similar bacterial flora, dominated by gram-negative bacteria, such as *P. gingivalis*.^{16, 17} LPS, a pathogenic factor of *P. gingivalis*, is involved in the onset of peri-implant and periodontal diseases.^{18, 19} Wang et al. reported that stimulation of HUVEC with *P. gingivalis*-derived LPS at concentrations >1.0 µg/mL resulted in enhanced *IL-8* and *ICAM-1* gene expression levels.²⁰ In addition, Xu et al. reported increased *ICAM-1* gene expression levels in HUVEC stimulated with 1.0 µg/mL of *P. gingivalis*-derived LPS.²¹ In this study, we established an experimental model of peri-implant mucositis by stimulating ARMEC with *P. gingivalis*-derived LPS, thus mimicking the peri-implant mucosa. The LPS concentration was set at 1.0 µg/mL to ensure the induction of an inflammatory response.

Vascular endothelial cells recruit leukocytes to sites of inflammation by expressing leukocyte chemotactic factors and adhesion molecules, such as *IL-8* and *ICAM-1*.^{8, 9} For example, Zitzmann et al. reported that the gene expression levels of leukocyte migration factors, such as *ICAM-1* and *E-selectin*, in alveolar ridge mucosal tissue is generally lower than that in gingival tissue.²² The present study revealed significantly lower *IL-8* and *ICAM-1* gene expression levels in ARMEC + LPS than in PDLEC + LPS, indicating lower levels of leukocyte migration in the peri-implant mucosa than in periodontal tissue. Interestingly, leukocyte migration is reportedly associated with tight junction cell adhesion.²³⁻²⁵ Srinivasan et al. quantified tight junction cell adhesion by measuring the TEER of various types of cells, including human pulmonary microvascular endothelial cells.²⁶ Furthermore, Dewi et al. analyzed the TEER of HUVEC and reported that higher TEER values corresponded to stronger cell adhesion and lower permeability.²⁷ Tight junction proteins include *Occludin*, which mediates interactions between adhesion molecules,²⁸ and *ZO-1*, which is necessary for *Occludin* accumulation at tight junctions.²⁹ Wang et al. reported that adding vascular endothelial cell growth factor to human brain microvascular endothelial cells resulted in decreased *ZO-1* and *Occludin* gene expression, thus weakening cell adhesion and increasing permeability.³⁰ In the present study, LPS significantly reduced TEER, as well as *ZO-1* and *Occludin* gene expression levels, in ARMEC. Additionally, ARMEC + LPS showed significantly higher TEER, as well as *ZO-1* and *Occludin* gene expression levels, than PDLEC + LPS. These results suggest that the peri-implant mucosa, with low leukocyte chemotactic factor gene expression levels and strong cell adhesion, may exhibit lower levels of leukocyte migration and less apparent signs of inflammation when inflammation occurs.

Various materials are currently used in dental treatments; however, few studies have reported on the impact of these materials on leukocyte migration and vascular endothelial cell adhesion. EMD not only acts on undifferentiated mesenchymal cells to promote periodontal tissue regeneration, but also stimulates the gene expression of leukocyte migration factors in vascular endothelial cells.¹³ Therefore, stimulating ARMEC with EMD may alter the inflammatory response in the peri-implant mucosa, allowing for the early detection of inflammation. For example, Andrukhov et al. reported a significant increase in *ICAM-1* gene expression in HUVEC stimulated with 100 µg/mL EMD.³¹ Additionally, Schlueter et al. stimulated human dermal microvascular endothelial cells with 10, 25, 50, and 100 µg/mL EMD and reported a significant increase in angiogenesis at an EMD concentration of 100 µg/mL.³² In the present study, EMD was used at 100 µg/mL to ensure an effect on vascular endothelial cells. EMD stimulation increased *IL-8* and *ICAM-1* gene expression levels, significantly decreased TEER, and decreased *ZO-1* and *Occludin* gene expression levels in ARMEC. Simultaneous stimulation with LPS and EMD enhanced *IL-8* and *ICAM-1* gene expression levels, suppressed TEER, and decreased *ZO-1* and *Occludin* gene expression levels compared to LPS stimulation alone. Furthermore, simultaneous stimulation of ARMEC with LPS and EMD did not show any significant differences in *IL-8*, *ICAM-1*, *ZO-1*, *Occludin*, and TEER compared to LPS stimulation of PDLEC. Therefore, the application of EMD to the peri-implant mucosa is hypothesized to activate leukocyte chemotactic factors and cell adhesion molecules, leading to increased vascular permeability. This, in turn, is believed to establish an immunostimulatory mechanism similar to that of periodontal tissue. This suggests that when the EMD-stimulated peri-implant mucosa is inflamed by plaque, neutrophils and macrophages will actively migrate to the inflamed area, potentially resembling the immune response of the periodontal tissue. Therefore, from a clinical perspective, applying EMD to the peri-implant sulcus during recall after implant treatment has been suggested as a means to establish an immunostimulatory mechanism in the peri-implant mucosa. This may activate the immune response and facilitate early detection of peri-implant mucositis.

Currently, research on the impact of EMD on endothelial cells is limited, and the underlying mechanisms remain unclear. Bertl et al. reported that EMD binds to TGF-β receptors on HUVEC.¹³ Additionally, TGF-β signaling is known to act through both Smad-dependent and Smad-independent pathways, influencing the immune response.³³ In this study, EMD, acting on ARMEC, is hypothesized to undergo similar signal transduction, contributing to the establishment of an immunostimulatory mechanism.

【Conclusion】

ARMEC obtained from the mucosa sites at which implants were to be placed, which are assumed to represent the peri-implant mucosa, showed lower leukocyte chemotactic factor gene expression and stronger cell adhesion than PDLEC, suggesting that signs of inflammation are less likely to appear in the mucosa than in the periodontal tissue. ARMEC stimulated with EMD exhibited increased leukocyte chemotactic factor gene expression and decreased adhesion, suggesting that an immune response had been induced and that signs of inflammation were likely to be increased. Based on the results of this study, applying EMD to the peri-implant mucosa during recall visits after implant treatment might alter these inflammation-prone tissues, aiding in early detection of peri-implant mucositis, allowing early treatment, and thus contributing to a favorable long-term prognosis.

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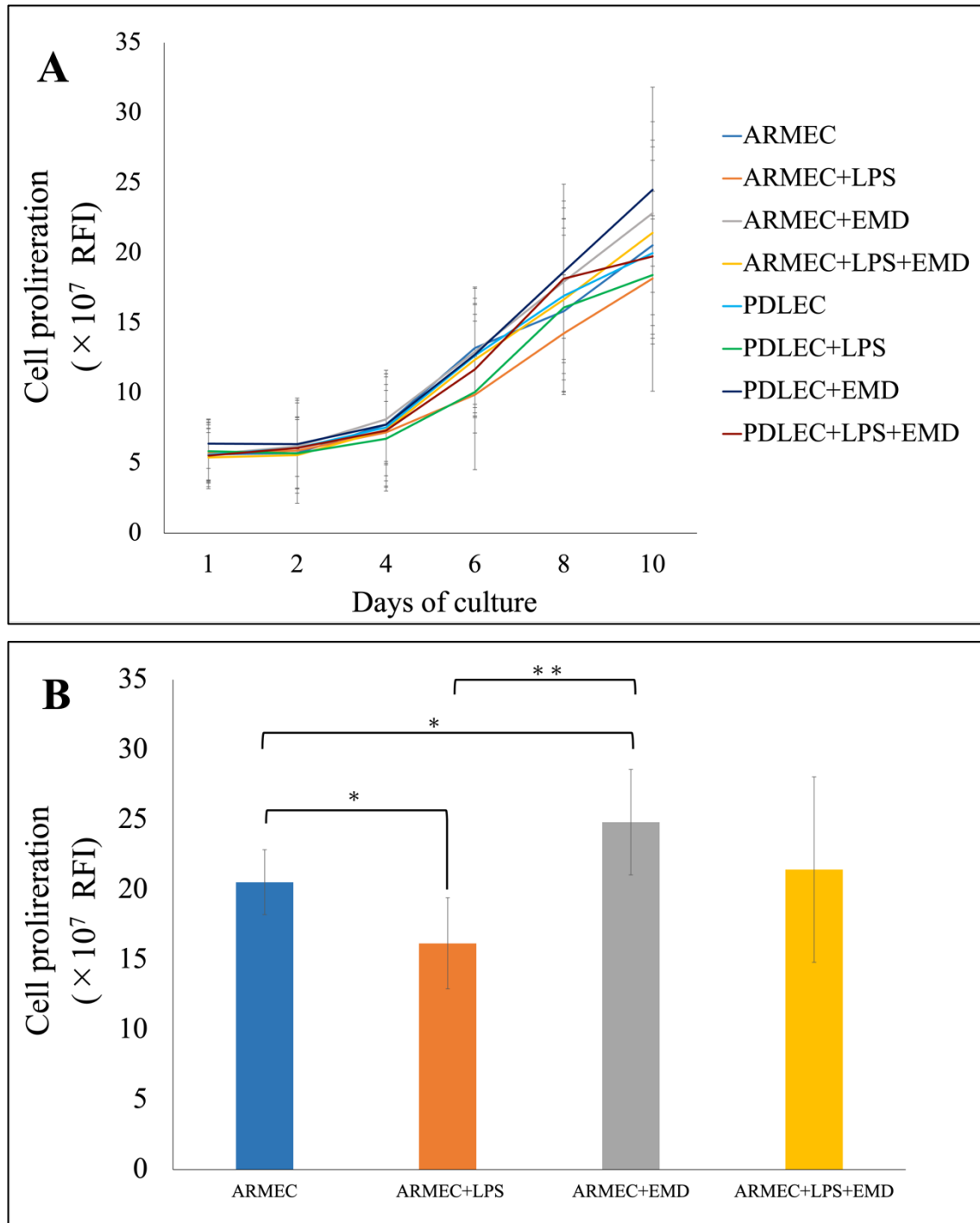


Fig. 1. Cell proliferation of ARMEC and PDLEC under the various treatment and control conditions. **A** The effects of LPS and EMD on the viability of ARMEC and PDLEC over 10 days. **B** Effects of LPS and EMD on the viability of ARMEC on day 10 of culture. (* $p < 0.05$, ** $p < 0.01$). **RFI** : relative fluorescence intensity.

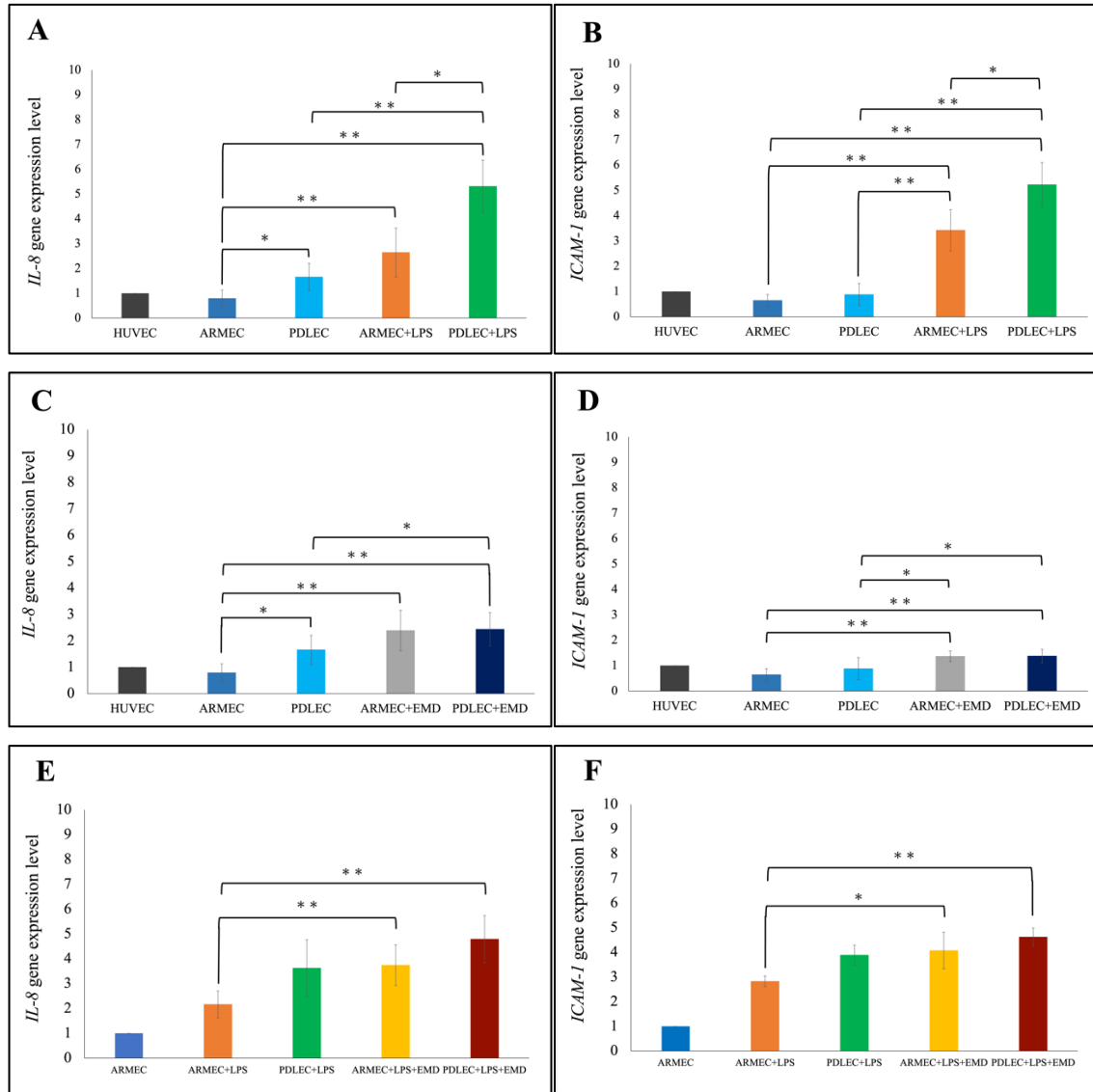


Fig. 2. Gene expression of leukocyte chemotactic factors and adhesion molecule (measured via *IL-8* and *ICAM-1* mRNA gene expression levels) in ARMEC and PDLEC under various treatment conditions in 6 h. RNA was extracted from stimulated ARMEC and PDLEC and subjected to reverse-transcription quantitative polymerase chain reaction for relative *IL-8* and *ICAM-1* gene expression analysis. Gene expression levels were presented relative to that in non-stimulated HUVEC at 6 h of culture in panels A-D and relative to that in non-stimulated ARMEC at 6 h of culture in panels E and F. (* $p < 0.05$, ** $p < 0.01$.)

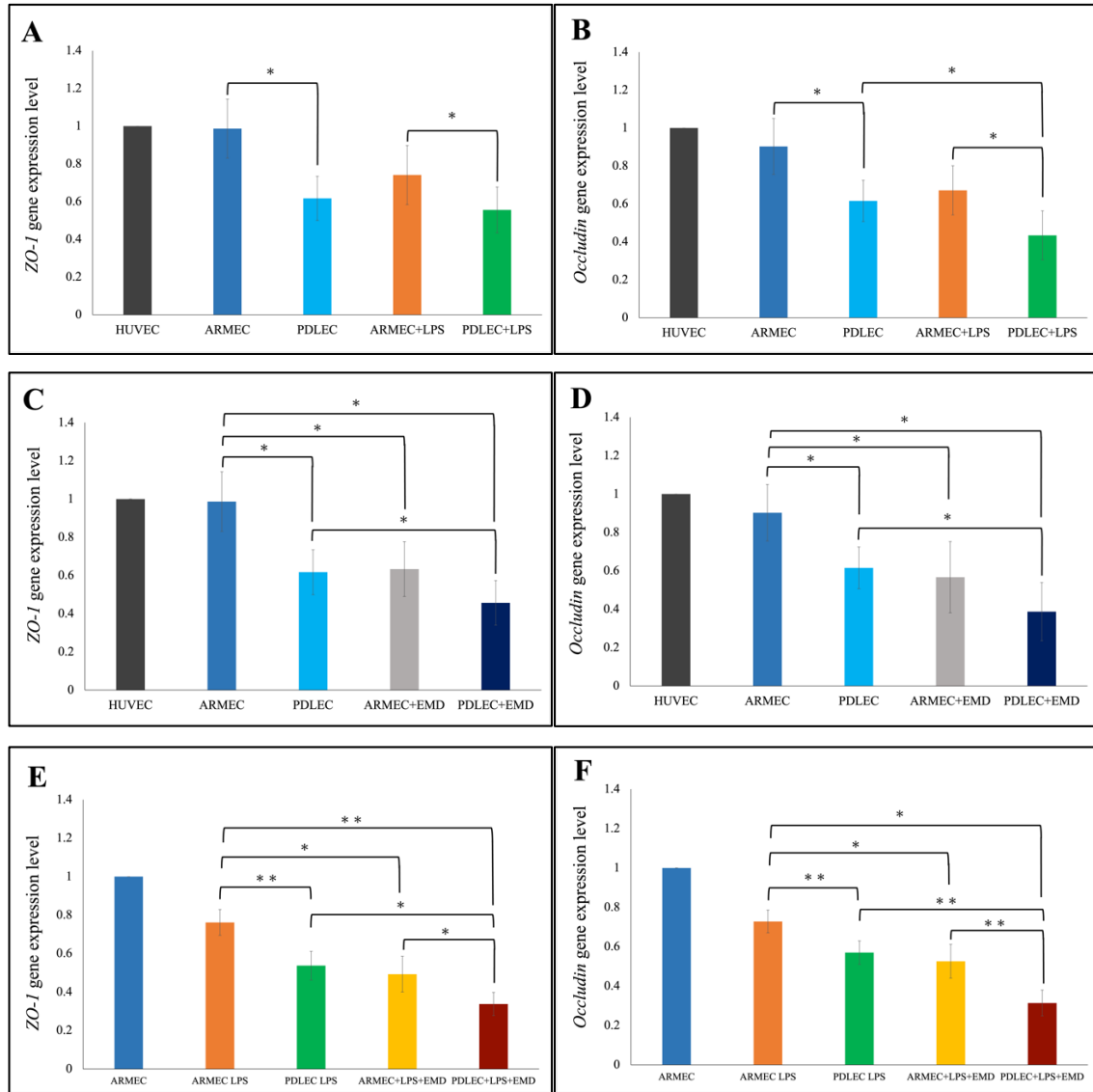


Fig. 3. Tight junction protein gene expression (measured using *ZO-1* and *Occludin* gene expression levels) in ARMEC and PDLEC under the various treatment conditions in day 6. RNA was extracted from stimulated ARMEC and PDLEC and subjected to reverse-transcription quantitative polymerase chain reaction for relative *ZO-1* and *Occludin* gene expression analysis. Gene expression levels were presented relative to that in non-stimulated HUVEC at day 6 of culture in panels A-D and relative to that in non-stimulated ARMEC at day 6 of culture in panels E and F. (* $p < 0.05$, ** $p < 0.01$.)

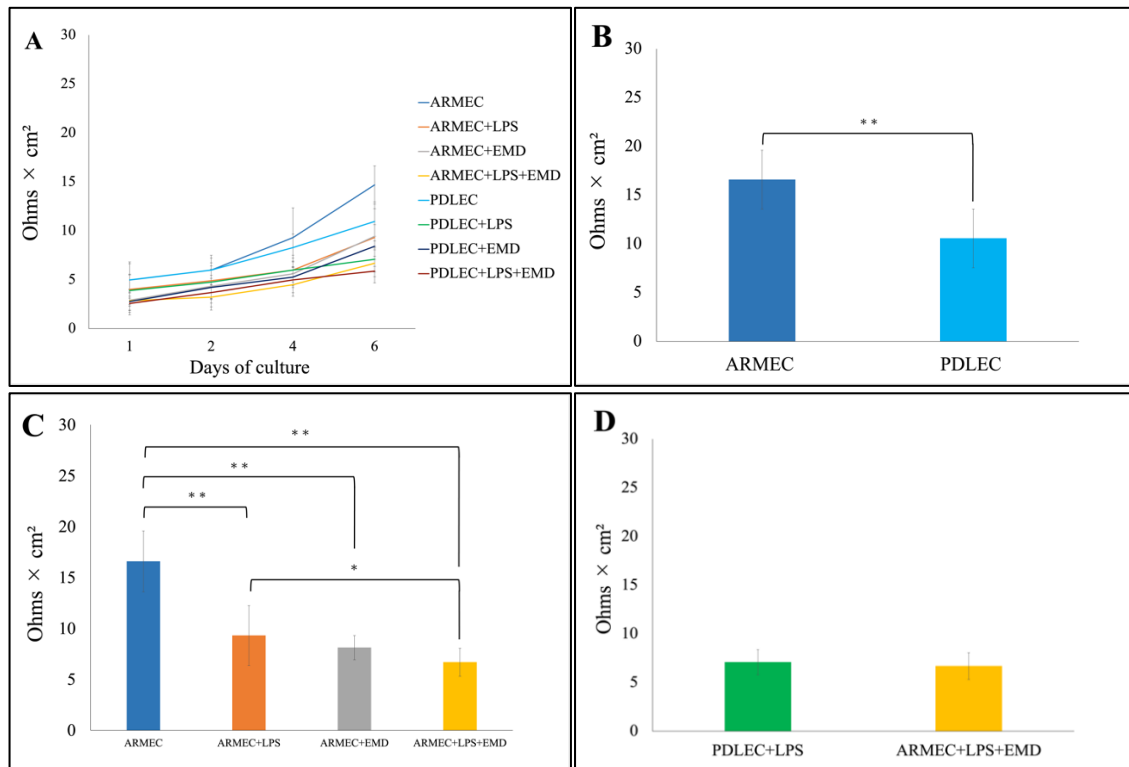


Fig. 4. TEER in ARMEC, and PDLEC under various treatment conditions. **A** The effects of LPS and EMD on the TEER of ARMEC, and PDLEC were measured over 6 days. **B** Cell-to-cell comparison in control on day 6 of culture (** $p < 0.01$, * $p < 0.05$). **C** Effects of LPS and EMD on the TEER of ARMEC on day 6 of culture(** $p < 0.01$, * $p < 0.05$). **D** Comparison of simultaneous stimulation of ARMEC with LPS and EMD and LPS stimulation of PDLEC on day 6.

Table 1. Sequences of the primers used in this study

| Gene | Primer Sequence 5'→3' | Accession number |
|-----------------|--|------------------|
| <i>GAPDH</i> | Forward : GCTCCCTCTTTCTTTGCAGC Reverse : CATGAGTCCTTCCACGATACCA | NM_001357943.2 |
| <i>ZO-1</i> | Forward : AGACAAGATGTCCGCCAGAG Reverse : CTGGTCCTCCTTTCAGCACA | NM_001355014.2 |
| <i>Occludin</i> | Forward : CTTCAGGCAGCCTCGTTACA Reverse : CCGCCAGTTGTGTAGTCTGT | NM_031484.4 |
| <i>IL-8</i> | Forward : CCACACTGCGCCAACACA Reverse : AACCTCTGCACCCAGTTTTC | NM_000584 |
| <i>ICAM-1</i> | Forward : ACGGAGCTCCCAGTCCTAAT Reverse : CTCCTTCTGGGGAAAGGCAG | NM_000201.3 |